A PRELIMINARY HISTOLOGICAL STUDY OF THE INSEMINATION REACTION IN DROSOPHILA GIBBEROSA[1]

HELEN TSUI-YING LEE[2]

Division of Entomology and Economic Zoology, University of Minnesota

The insemination reaction was first described by Patterson (1946) in Drosophila, and has been subsequently studied in numerous species of the genus by Wheeler (1947). The reaction occurs strongly, weakly or not at all depending on the species being studied. When present as a strong reaction (as in *Drosophila gibberosa*) it consists of a swelling of the vagina to three to four times the normal size following mating. This swelling is brought about by the secretion of fluid from the vaginal epithelium in response to some stimulus supplied by the semen; in at least some respects it resembles an immunological reaction. Commonly, especially in reactions resulting from the mating of different species, a more or less opaque mass develops in the lumen of the vagina; this is termed the "reaction mass."

Previous work has been limited to the observation of gross dissections and the determination of genetical results. The present study was initiated to extend the study to histological structure and to determine the specificity of the reaction, and perhaps the steps involved, by the artificial insemination of materials other than semen.

Materials and Methods

Drosophila gibberosa was selected because of its large size, availability and ready rearing on standard media. Since adults of this species do not mate until nine to fourteen days after emergence, males and females were kept separately in humid milk bottles and fed bananas or a yeast-honey mixture during this period.

Mating can be obtained with single females and several males in separate containers, but for the purposes of this work mass methods were more satisfactory. Ten to twenty females and a similar number of males were placed in glass containers of approximately one pint capacity. The containers were lined with wet filter paper to maintain a high humidity. Reasonable numbers of matings occurred within 30 minutes. Mating pairs were isolated by inverting small vials over them, the vials being numbered corresponding to notes kept on each pair.

The abdomens of mated females were cut off and fixed in Brazil's (alcoholic Bouin's) fluid at 0, 5, 15, 30, 60, 120, 180, 240, 300, and 360 minutes after the beginning of mating. In some tests intervals as long as 11 hours were used. They

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[2] Present address: Sun Yat Sen University, Canton, China.

were then washed, dehydrated, sectioned in paraffin (Tissuemat), and stained with Delafield's haematoxylin and eosin or acid fuchsin and congo red by routine methods.

For artificial insemination the desired organs were dissected free in saline,[3] cleaned of adhering tracheae and other tissues, and ground into a brei in saline using a small mortar and pestle. Capillary glass tubes sealed onto ordinary syringe needles were drawn to desired fineness and used for injecting into the vaginae. Etherized females were placed ventral side up on the stage of a binocular microscope, held gently with forceps, and the needle (preferably held in an adjustable mechanical stage) manipulated through the gonopore into the vagina. In the present preliminary work, the injections were not quantitative but were roughly the same volume. Artificially inseminated females and ones injected with non-semen experimental fluids were prepared for histological examination in the same manner as the normally mated females.

Fluids injected into the lumen of the vagina artificially include:

1. Yeager's cockroach saline;[3] this has a tonicity equal to a 1.3 per cent solution of NaCl.
2. 2 per cent NaCl solution.
3. 2 per cent gelatine in cockroach saline solution.
4. brei of testes in saline solution.
5. brei of male accessory glands in saline solution.
6. brei of male accessory glands plus testes in saline solution.
7. brei of entire male fly in saline solution.

The volume of epidermal cells of the vagina was estimated by making camera lucida drawings of each section on calibrated graph paper, adding up the number of squares occupied by the drawings (estimating partial ones), and multiplying by the thickness of the sections. Probably a rather high percentage of error is involved in these estimates, but since the differences to be discussed are differences of several magnitudes the errors would not seem serious. It should be remembered, however, that all areas and volumes represent measurements on sectioned material; the figures are doubtless smaller than life size but should be accurate for relative sizes.

Results

1. Macroscopic features of vaginae of virgin females: The term vagina in insects has been variously used. Snodgrass (1935) regarded the vagina as that part of the female reproductive tract which extends posteriorly from the base of the median oviduct to the ovipositor. In dipterous insects, Pantel (1910) referred to it as the uterus (Townsend, 1911). Nonidez (1920) divided this region into two parts, a uterus proper and a vagina. For Drosophila, Patterson (1947) followed the terminology of Snodgrass but added the term "vaginal pouch" for the portion of vagina which extends antero-ventrally. During the course of insemination reaction, the wall of the vaginal pouch is capable of great distention, the wall of the vagina remains unchanged. For descriptive convenience, the terminology of Patterson was adopted in the present study.

[3] Yeager's cockroach saline: NaCl, 10.93 grams; KCl, 1.57 grams; $CaCl_2$, 0.85 gram; $MgCl_2$, 0.17 gram; $NaHCO_3$, 0.17 gram per liter of double distilled water.

In *Drosophila gibberosa* the vagina is a whitish pear-shaped body, situated at the posterior end of the abdominal cavity just beneath the rectum. The vaginal pouch is about one-third as large as the vagina and is situated antero-ventrally to the origin of the parovariae and ventral receptacles. Both vagina and vaginal pouch are provided with a well-developed muscular wall and are without significant demarcation externally. The gonopore is enclosed between two valves of the ovipositor which are located at the posterior end of the abdomen externally.

2. Microscopic structure of vaginae of virgin females: The vaginal wall is composed of three distinct layers. The innermost layer is a transparent thin non-cellular cuticular intima which is more prominent in the vagina than in the vaginal pouch. Apparently it was the endocuticular portion of this that was called a separate fibrous layer by Nonidez (1920) in *Drosophila melanogaster*. Second, there is a single layer of cuboidal epithelial cells, the nuclei of which are more or less oval, located usually in the middle of the cells and generally with distinct chromatin threads and one or two nuceoli. The epithelial layer is much thicker than the intima: it is slightly thinner on the wall of vaginal pouch and with more scattered and sparse cells as compared with aggregated and dense ones of the vagina. The epithelium and the intima are thrown deeply into many folds within the lumen, especially so on the wall of the vaginal pouch; thus the layers of cells appear to be superimposed on others in the section. Third, externally the vagina is lined by a thick muscular layer which is much thinner and sparser on the wall of the vaginal pouch. Presumably a basement membrane and some other slight connective tissue elements are present but these are not sharply demarcated. The volume of epithelium measured from three sets of serial sections averages 1,270,000 cubic microns.

3. Macroscopic features of vaginae of mated females: Immediately after mating the vaginal pouch begins to increase in size. In some cases the vaginal pouch reaches its greatest distention about two and one half hours after mating; in other cases four hours is required to attain the greatest distention. The size at greatest distention is about four or five times as large as the normal one. The actual size attained is the sum of (1) initial size, (2) amount of semen, (3) amount of material added to semen, and (4) any changes that occur in the vaginal wall. These will be discussed later.

The wall of the vaginal pouch becomes very thin and transparent. The vagina only slightly increases in size. Within the vaginal pouch an opaque reaction mass can be seen under the microscope. The vaginal pouch begins to contract after reaching its greatest distention and the opaque reaction mass also gradually decreases in size. It returns to original size and form about five hours after mating.

4. Histology of vaginae of mated females: As the wall of vaginal pouch is gradually distended after mating, its epithelial cells become flatter and flatter and the folds within the lumen finally disappear. The intima also becomes thinner. The muscular layer becomes very thin and the muscles widely separated. The vagina is less distended; its epithelial cells remain cuboidal. As a rule the nuclei of epithelium of reacting vaginae are generally more dense as compared with those of the virgin female and without the diffuse appearance of chromatin threads; the nuclei are frequently elongated and generally located at the base of the cells. The cell volume increases to about four times the normal at its greatest distention; it averages 4,800,000 cubic microns from the measurement of three sets of serial sections of vaginae.

After reaching its greatest distention, the vagina gradually returns to its normal histological structure and about five hours after mating there is no significant histological difference from that of the virgin female. The reaction mass is composed of spermatozoa and substance of acidophilic staining property; its increase and decrease of size keeps well in pace with the distention and contraction of the vagina. The entering of spermatozoa into the ventral receptacle can be traced in the sections.

5. Macroscopic features of vaginae of artificially inseminated females: Testes brei in saline, male accessory gland brei in saline, testes plus male accessory gland brei in saline, entire male fly brei in saline, cockroach saline, 2 per cent NaCl and 2 per cent gelatin in saline were used for artificial insemination. The results are different for different injection materials.

After the injection of testes brei the vaginae undergo macroscopic changes similar to those of the normal mated female. The same feature is found in the vaginae of testes plus male accessory gland injections but the distention is of lesser degree. Injection of saline also brings about the same amount of distention. The injection of male accessory gland brei, entire male fly brei, 2 per cent gelatin and 2 per cent NaCl may bring about an unsignificant increase of vaginal size by mechanical distention of fluid injected into the lumen, but the vaginal pouch does not thin out as in other experiments.

6. Histology of vaginae of artificially inseminated females: The vagina of testes brei injections undergoes a histological change similar to that of the mated female as far as the epithelium, intima, muscles and reaction mass are concerned. The volume of epithelial cells also increases; an average of measurement of two vaginae has the same figure as that of the mated female, that is 4,800,000 cubic microns.

The vagina of testes plus male accessory gland brei injections undergoes a similar histological change to that of the previous case, but the cells on the wall of the vaginal pouch do not thin out so extensively. The reaction mass is somewhat smaller in size, but the significance of this is open to some question, as will be discussed later.

The vaginae of male accessory gland brei injection, whole male brei injection and 2 per cent gelatin injection did not show any significant histological change.

The vaginae injected with saline (which apparently is hypotonic) undergo a peculiar histological change, in which the vaginal pouches thin out moderately with the outer muscle layer retained. Unlike the nuclei of the vaginal epithelium of the mated female, the nuclei are generally of diffuse appearance. Th cytoplasm is generally more lightly stained by eosin as compared with that of the virgin or mated female. Although the vaginae are distended moderately there is no reaction mass found in the lumen. The volume of cells also increases, but to a lesser degree, average of measurement of two sets of serial sections is 3,420,000 cubic microns.

Contrary to the results from saline injection, the vaginae after injection of 2 per cent NaCl does not exhibit significant distention. The cytoplasm of the vaginal epithelium is generally heavily stained by eosin and significant shrinkage is shown at certain areas. The nuclei are also slightly shrunken. The cell volume does not increase, an average of measurement of two sets of serial sections is 1,311,000 cubic microns which is close to the figure of virgin female.

The reaction of vaginae to different kinds of inseminations is summarized as follows:

Kinds of insemination	Increase of vaginal size	Increase of volume of vaginal epithelium	Reaction mass plus intense staining
Normal mating	++	++	+
Testes in saline	++	++	+
Testes plus accessory glands in saline	++	++	+
Saline (hypotonic?)	+	+	−
Male accessory glands in saline	+	0	0
Entire male fly in saline	+	0	0
2% gelatin in saline	+	0	0
2% NaCl (hypertonic?)	+	0	0

The volume of epithelial cells of vaginae of virgin females, mated females and females artificially inseminated with testes brei, saline and 2 per cent NaCl were measured. As stated under the MATERIALS AND METHODS, these figures are relative rather than absolute because they were made on sectioned material which unquestionably shrunk somewhat during the course of preparation. The average figures for the volume of cells are tabulated as follows:

Type of female	The volume of vaginal epithelium (in cubic microns)	Average (in cubic microns)
Virgin female	1,568,116	1,270,000
	1,285,274	
	1,046,470	
Mated female	4,056,381	4,800,000
	4,895,334	
	5,448,870	
Female of testes injection	3,502,842	4,800,000
	6,097,545	
Female of saline injection	3,433,653	3,420,000
	3,596,533	
	3,234,726	
Female of 2% NaCl injection	1,407,400	1,311,000
	1,215,358	

DISCUSSION

Patterson (1946) described the presence or absence of the insemination reaction in thirty-five species of Drosophila. In 1947 he made an experiment of homogamic matings of eight species and twenty-six crosses of the species of the genus Drosophila. He found that in homogamic matings it takes five to ten minutes after mating for the sperm to move forward to the point of origin of the ventral receptacle and several hours to enter the ventral receptacle. In heterogamic matings similar movement of sperm take about forty-five minutes and the spermatozoa frequently form a tangled mass about the opening to the ventral receptacle. He pointed out that this has the effect of retarding or inhibiting the entrance of sperm into the lumen of the receptacle. In many interspecific crosses, the sperm never fertilize the eggs due to the deleterious effect of the insemination reaction and the resulting reaction mass. The egg may disintegrate in the cavity of the vagina.

Patterson (1946) inferred that the insemination reaction may have three possible functions: It may have the effect of (1) preparing the reproductive tract for the fertilization mechanism, especially in the case of homogamic matings: and it may have a direct bearing on the problem of speciation both (2) by deterring genic interchange and (3) by limiting the number of matings an individual female may have.

As regards the induction of an insemination reaction among homogamic matings of *Drosophila aldrichi*, Patterson (1947) found in one case that the vagina had already reached its maximum reaction, although the female was dissected before the termination of mating. Yet, in the distended vagina or ventral receptacle there was not a single spermatozoon. From this, he assumed that the spermatozoa are not the active agent to bring forth the insemination reaction. He undertook a convincing experiment to support his assumption. In the backcross of the sterile F_1 male from the *mulleri/mojavensis* with *mulleri* females, the vagina is slightly distended. Since the semen of this F_1 hybrid male is sperm free, the result demonstrates conclusively that the presence of spermatozoa are not necessary for the induction of the insemination reaction.

The knowledge about the exact composition of the semen of Drosophila is meager. According to Nonidez (1920) the accessory glands secrete a sticky fluid with floating refractive granules and this fluid becomes mixed with the spermatozoa from the testes to form the ejaculate. However, although the spermatozoa were found not necessary to induce reaction, at least in the above experiment, Patterson does not conclude that the secretion of the male accessory gland has such a function. He stated that the semen of F_1 hybrid of *mulleri/mojavensis* is sperm-free but does not exclude the possibility that degenerative products from the abnormal testes may constitute a part of the semen. This must await fractionation of semen into its components. Patterson points out superficial similarities to immunochemical reactions but direct evidence is lacking.

Wheeler (1947) classified seventy-eight species belonging to the genera Chymomyza, Scaptomyza and Drosophila of the family *Drosophilidae* into three classes according to the degree of insemination reaction in homogamic matings. Class 1 is composed of those forms in which no reaction is apparent; he lists seventeen species including the common *D. melanogaster*. Class 2 contains those in which a slight or moderate reaction develops; he lists forty-one species. Class 3 contains those in which there is a considerable enlargement of the vagina and formation of a dense opaque reaction mass; he lists twenty species. He also considered the problem of evolution of Drosophila from the viewpoint of insemination and the possible function of an insemination reaction in homogamic matings. As indicated by Patterson, it may have the effect of preparing the reproductive tract for the fertilization mechanism in homogamic matings. It may be correlated with the slower movement of the spermatozoa into the ventral receptacle of the immobilization of the sperm possibly due to the greatly increased viscosity of the reaction mass.

In the present study the size of the vagina was found to increase in various degrees after natural and certain artificial inseminations. The increase of size at the maximum reaction was greater for natural insemination and testes brei injections than for the other materials. The reaction mass is present in the vagina of natural insemination, testes brei injection, testes plus male accessory gland brei injection. The vaginae which increased in size significantly also showed an increase of the

volume of epithelial cells, but degree of increase is not the same in all cases. The vaginae of natural insemination and testes brei undergo the maximum increase of cell volume, a lesser degree was found in the testes plus accessory gland brei injections and a still lesser degree in the saline injections.

At first sight it may seem surprising that the vaginal epithelial cells increase in volume, especially since distention of the vagina makes the vaginal wall become thinner. But it should be remembered that the vaginal wall is only an invaginated part of the general body integument, and that it is well known that the epidermis of arthropods increases greatly (common range 2–5 times in thickness) during the period when a new cuticle is being deposited (Wigglesworth, 1939). The reaction of the vaginal wall, then, seems quite in keeping with reactions to be expected of arthropod epidermal cells.

The vaginae with a reaction mass have their epithelial nuclei somewhat dense in appearance. In other cases, even though the vagina may increase significantly in size, as in saline injections, the reaction mass is absent, the nuclei generally show diffusely oriented chromatin threads as those of the virgin female, and the cytoplasm is more lightly stained.

In mated females the vagina increases in size after mating (Patterson) and therefore the reaction mass must contain both semen and material added to it. Presumably the material added to the semen is secreted by the vaginal epithelium but origin from associated organs is not excluded. In addition the cells of the vaginal epithelium increase in size and stainability; therefore they too have material added to them. In response to the stimulus from the semen, these cells must absorb material from the blood (and perhaps alter this metabolicly). If these cells secrete the material into the lumen of the vagina, then they must absorb from the blood enough to account for the increase in both the reaction mass and in the cells. Obviously there is a strong reaction by the cells to the stimulus afforded by the presence of semen (or some component in the semen).

The reaction brought about by mating and by testes brei or testes plus male accessory gland brei injection reveals all the features just mentioned. However, in the specimens injected with saline, although the size of the vagina and the volume of epithelial cells increase, the nuclei are loose and the cytoplasm is lightly stained and the reaction mass is never present. From the evidence that the cytoplasm is lightly stained it is assumed that increase of cell volume is simply the result of the uptake of water from the injected saline on account of the lower osmotic pressure of the saline used as compared with the protoplasm of the epithelium. In order to make a more accurate comparison of the staining susceptibility of cytoplasm of vaginae after various treatments, sections of vaginae from a mated female, a female of saline injection and a virgin female were arranged on the same slide so as to obtain a uniform staining process. The results show that the cytoplasm of vaginal epithelium from the mated female is more heavily stained by hematoxylin and eosin than that from the female of saline injection, while that of the virgin female has a staining susceptibility intermediate between those two. Obviously, the cytoplasm of vaginae injected with saline is diluted and the dilution is almost certainly due to the uptake of water from the saline solution injected. The contraction of vaginal epithelial cells by injection of 2 per cent NaCl contributes further support to this

assumption, because the osmotic pressure of 2 per cent NaCl is generally higher than that of the protoplasm of insects.

Since the injection of male accessory glands did not induce any insemination reaction in the present data, it is assumed that the secretion of the male accessory gland is not the substance to initiate the reaction. Quantitative effects are not yet precluded but it seems most reasonable to conclude tentatively that the reaction is due to some semen component derived from the testes. As Patterson presented some evidence that the spermatozoa are not the active agent to induce reaction, the inducing substance may be a factor in the testes besides spermatozoa. The active component is not yet known, and we have only two clues about it: (1) it is probably from the testes, and (2) its action suggests protein.

Summarizing the material presented in this discussion: The insemination reaction can be broken down into five component parts: (1) an increase in size of vagina, (2) an increase in volume of epithelial cells, (3) the presence of a reaction mass, (4) a dense appearance of nuclei, and (5) heavily stained cytoplasm. To a certain extent these are experimentally separable. All are produced by mating and by the injection of brei containing testes. (1) plus (2) can be produced by hypotonic saline solution; (4) plus (5) can be produced by hypertonic saline solution; (1) alone is produced by osmotically inactive solutions. It would seem likely that (3) alone could not be produced, *i.e.*, that the reaction mass necessitates the other component steps, but this cannot be proved until the active agent is available for artificial insemination.

Summary

1. Macroscopic features and microscopic structure of vaginae of *Drosophila gibberosa* Patterson and Mainland are described in virgin females, mated females, females with testes injection, male accessory gland injection, testes plus male accessory gland injection, whole male brei injection, 2 per cent gelatin injection, saline injection and 2 per cent NaCl injection.

2. The wall of the vagina consists of intima or cuticle, epithelium and muscle layers.

3. The insemination reaction occurs in vaginae of mated females, females with testes injections, and testes plus male accessory glands injections. The reaction consists of five characteristic features: (a) increase of size of vagina, (b) increase of volume of epithelial cells, (c) presence of reaction mass, (d) dense appearance of nuclei, and (e) more heavily stained cytoplasm. Some but not all of these features have been produced separately by artificial insemination with experimental solutions.

4. The increase in volume of the vaginal epithelial cells is compared with the known increase in volume of epidermal cells during deposition of a new cuticle.

5. Cell volumes can be altered by the injection of hypertonic and hypotonic saline solutions as shown by both volume and intensity of staining.

6. The reaction of vaginae of mated females reaches its maximal distention two and a half to four hours after mating. Then it decreases in size gradually and returns to its normal size five or six hours after mating.

7. The reaction mass within the vagina is composed of spermatozoa and substances of acidophilic staining property. The increase and decrease of the size of

the reaction mass during the course of the reaction keeps well in pace with the size of the vagina.

8. The insemination reaction of vagina as demonstrated by Patterson (1947) seems not induced by spermatozoa, and the present data show that it is not induced by male accessory glands either. Since the semen of Drosophila consists of secretion of accessory glands, spermatozoa and some substances from testes besides spermatozoa, it is suggested that the agent which induced the insemination reaction is most likely some factor from the testes other than the spermatozoa.

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